Document Number: **SASoM/METHOD/078.v4**

Title: **Transduction of GECKO Library in MiaPaCa2 cells**

Version: **v4**

Author: **Paul Reynolds**

Effective from:	19/04/2021
Valid to:	19/04/2023

SOP History		
Number	Date	Reason for Change
v1	02/04/2015	Original
V2	02/04/2017	Update
V3	02/04/2019	Update
V4	19/04/2021	Update

1.0 Purpose –

This SOP describes the current procedure for transduction of GeCKO library in MiaPaCa2 cells for loss of function genetic screen in Laboratory 248 at the St Andrews School of Medicine (SASoM).

2.0 Scope –

This SOP applies to all staff in the SASoM involved in .genome wide loss of function screen using GeCKO library in the pancreatic cancer cells MiaPaCa2.

3.0 Responsibilities –

All staff are responsible for ensuring that the methods are followed in accordance with this SOP.

All staff must have read and signed the relevant risk assessment documents before performing this procedure.

4.0 Procedure –

SPECIFICATIONS :

- GeCKO Library A+B: 122417 sgRNAs total
- MOI (Multiplicity of Infection) = 0.3 (~1 virus/cell)
- Coverage ~ 300 fold (~320 cells/sgRNA)
- Number of MiaPaCa2 cells to infect: (sgRNAs*coverage)/ MOI
  $=1.3\times10^7$ cells
- Number of viral particles needed: number recipient cells*MOI
  $= 3.9\times10^7$ viral particles
- DNA input for PCR: sgRNAs*coverage*mass DNA in10^6 cells
  =260µg DNA
- Number of cells to harvest to get >260µg DNA = 6×10^7 cells

PART A: LENTIVIRAL PRODUCTION IN 293T CELLS:

Early passage HEK293T cells (designated 293T(SD)) should be used for lentiviral production. Later passage cells will be inefficient at producing virus.

Day 1 - Plating Cells:

Prepare 2 plates of 4×10^6 HEK293T cells in 10cm T/C dishes (in a volume of 10mL).

Day 2 - Cell Transfection:

Transfect 1 plate with “library A” and 1 plate with “library B”:

Put 50mL aliquot tube containing OPTIMEM into the water bath to pre-warm. Then add 1.5mL of this OPTIMEM into a 15mL tube (1 tube for each petri dish of cells).

Add the appropriate amount of Mirus LT1 to the OPTIMEM as required (according to the table). Gently pipette Mirus LT1 directly into the media without touching the plastic of the tube. (NEVER vortex). Wait 20 minutes.

While waiting, make up DNA mixture in sterile eppendorf tube as required (again according to the table below). Use pVSV.g and psPAX2 for packaging plasmids (2nd generation packing) plus the expression plasmid of interest pLentiCRISPRv2.

After the 20 minutes has elapsed, add DNA mix to the Mirus LT1-OPTIMEM solution. Gently pipette. (NEVER vortex). Wait 30 minutes.

Add DNA-Mirus LT1-OPTIMEM solution dropwise evenly across the surface of a 10cm T/C plate of HEK293T cells containing 10 mL DMEM/10%FBS/1% PS. Place in incubator labelled "viral' overnight.

Vector	# of plates to make	pVSVg (uL) [pSD11]	psPAX2 (uL)	DNA (uL)	Mirus LT1 (uL)	DMEM	Volume /plate
pLentiCRISPRv2 A	1	2.0	3.0	4.0	27.0	1.5	1.5
pLentiCRISPRv2 B	1	2.0	3.0	4.0	27.0	1.5	1.5

Day 3 - Change medium to reduce volume and increase viral particle concentration:

Change media at <24 hrs post-transfection, adding 4 mL DMEM / 10%FBS / 1% PS. From this point onwards, live virus is being produced – take appropriate safety measures!

Use the "viral" safety cabinet and "viral" labelled equipment for all manipulations.

Day 4 - Collect viral particles

Collect media-containing viral particles from cells, 48hr post-transfection into a 15mL tube.

CAREFULLY add 4 mL of fresh DMEM/10%FBS/1% PS to the plate (these cells detach easily). The viral particle medium should be a clear but slightly orange in colour (cloudy = contamination).

Store 15mL tube in a box labeled "virus" in the fridge in 248K.

Day 5 - Collect viral particles

Collect media containing viral particles from cells, 72hr post-transfection. The viral particle medium should be a clear and slightly orange in colour (cloudy = contamination).

Combine with viral particles collected from cells 48hr post-transfection.

Pass pooled 48hr and 72hr viral particle media through a 0.45µm filter using a 10ml syringe into a 50mL tube containing 8µg/ml polybrene (1000x stock). This step removes cellular debris.

Dispose of HEK293T cell plates by autoclaving.

PART B: LENTIVIRAL INFECTION/ TRANSDUCTION OF ADHERENT RECIPIENT CELLS.

Day 4 - Plate 'recipient' cells:

Plate 13×10^7 MiaPaca2 cells split into 10 T-150 flasks (~1.3×10^7 cells/flask) with 50 ml DMEM/ 10%FBS / 1% PS

Day 5 - Collect viral particles and infect recipient cells:

Step described above.

Infect recipient cells:

Viral titer~ 4×10^7 viral particles /ml
MOI=0.3
Take MiaPaca2 cells plated on day 4 (70% confluent~2×10^7 cells) and infect each flask with 6×10^6 viral particles =150µl viral solution (75 µl virus A+ 75 µl virus B):

Dilute each virus solution, A and B, to 1/100 by serial dilutions:
Add 1ml virus solution to 9ml medium =1/10 (1)
Add 10ml virus solution (1) to 90ml medium =1/100 (2)

Pool diluted virus solution (2) A with virus solution (2) B

Aspirate medium from the recipient cell flasks and add 15ml of pooled viral solution in each flask. Give a little rock across to spread the viral particles.

Place flasks in the "viral" incubator (37°C) for 4 hours (gently rocking occasionally to aid infection). After 4 hours, aspirate viral particle media to liquid viral waste.

Add 20ml culture medium in each flask and aspirate to wash the remaining viral particles then replace with 50ml culture medium.

Incubate for 48h in "viral" incubator

Day 7 - Select recipient cells with puromycin:

After 48h post-infection, select cells with puromycin
Puromycin works most efficiently on floating, trypsinized cells.

Therefore, aspirate media, add 10mL of PBS, aspirate PBS and incubate at 37ºC with 4mL TrypLE Express (Invitrogen) until cells are detached (about 10min). Add 6mL of culture media and transfer to 50mL tube.

Centrifuge to pellet cells (1,200 rpm for 3 min).

Add 40mL culture media to a T150 flask and add 10µl of 10mg/ml puromycin to get 2µg/ml in 50ml. Do this by dropping with a micropipette from the top of the flask. Do not contaminate the flask by placing a micropipette into the flask.

Resuspend cells in 10mL culture media and transfer to the T-150 flask. Incubate at 37ºC in the "viral" incubator.

Maintain cells for 7 days (change medium every 2-3 days) to let time for genomic changes, transduced cells should be successfully puromycin selected after 4 days.

After 7 days, trypsinize and pool cells from the different flasks.

Count cells with haemocytometer and freeze down 4 pellets of 6×10^7 cells (~396µg DNA) for baseline sequencing.

Split the rest of the pooled cells into drug conditions with a minimum of 2.6×10^7 cells for each condition:

-2 T150 flask 50ml DMEM + 15nM Gemcitabine
-2 T150 flask 50ml DMEM + 65nM Acelarin S (NUC-1031 Sp)
-2 T150 flask 50ml DMEM + equal volume DMSO (= control)

Maintain cells for 28 days in these conditions.

Harvest and freeze down 6×10^7 cells for sequencing at day 14, 21 and 28 post-treatment.

PART C: PREPARE SAMPLES FOR NEXT GENERATION SEQUENCING:

Thaw frozen cell pellets.

Extract genomic DNA with a Qiagen Blood and Cell culture Midi kit (SASoM/METHOD/077: Genomic DNA purification using QIAGEN Midi prep kit).

Quantify the extracted DNA with QubiT fluorometer (SASoM/METHOD/079: DNA quantification using QubiT fluorometer 2.0 for GeCKO library).

Perform PCR in 2 steps (see PCR protocol for lentiCRISPRv2 next generation sequencing)

5.0 Personal protection -
A Howie laboratory coat and lab gloves must be worn at all times.

6.0 Spillages -
Always clean up any spills immediately after use, only you know what you have spilt and are aware of its hazard.

7.0 Training -
All users must be fully trained in cell culture. All users must be fully trained in the handling of virus cultures.

8.0 Related documents –

8.1 Risk assessments:
RA/BIOL/001 - Handling of bacteria

RA/BIOL/004 - Tissue culture

RA/GM/002 - Viral-mediated gene delivery into mammalian cells

8.2 SOPs:
SASoM/METHOD/075 - GeCKO lentiCRISPRv2 library DNA amplification in Lucigen bacteria.

SASoM/METHOD/077 - GeCKO genomic DNA purification Qiagen Midi kit.

SASoM/METHOD/078 - Transduction of GECKO Library in MiaPaCa2 cells.

SASoM/METHOD/079-DNA quantification using QubiT fluorometer 2.0 for GeCKO library.

SASoM/METHOD/080- PCR for GeCKOv2 library preparation for Next generation sequencing.

SASoM/METHOD/081-sgRNA Target Guide Sequence Cloning into pLentiCRISPRv2.

SASoM/METHOD/082-AMPure PCR purification for GeCKOv2 NGS.

SASoM/METHOD/083-Quality control of GeCKOv2 for NGS using Agilent Bioanalyzer system.

9.0 Approval and sign off –

Author:

Name: Paul Reynolds

Position: Principal Investigator

Signature:  Date: 19/04/2021

Management Approval:

Name: Peter Mullen

Position: Research Fellow

Signature:  Date: 19/04/2021

QA release by:

Name: Peter Mullen

Position: QA Manager

Signature:  Date: 19/04/2021

STANDARD OPERATING PROCEDURE

Please sign below to indicate you have read this S.O.P and understand the procedures involved.

NAME	POSITION HELD	SIGNATURE	DATE